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# Direct Peptide Profiling by Mass Spectrometry of Single Identified Neurons Reveals Complex Neuropeptide-processing Pattern*

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**Ka Wan Li‡§, Robert M. Hoek‡, Frank Smith¶, Connie R. Jiménez‡, Roel C. van der Schors‡, Peter A. van Veelen‖, Su Chen**, Jan van der Greef‖, David C. Parish‡‡, Paul R. Benjamin¶, and Wijnand P. M. Geraerts‡**

*From the ‡Graduate School Neurosciences Amsterdam, Research Institute Neurosciences Vrije Universiteit, Faculty of Biology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands, the **Department of Chemistry, University of Warwick, Coventry CV4 7AL, United Kingdom, ‖TNO-IBC, Centre for Structure Elucidation and Instrumental Analysis, P. O. Box 360, 3700 AJ Zeist, The Netherlands, the ¶Sussex Centre for Neuroscience, School of Biological Sciences, University of Sussex, Brighton, East Sussex BN1 9QG, United Kingdom, and the ‡‡Unit of Metabolic Medicine, St. Mary's Hospital Medical School, Paddington, London W2 1PG, United Kingdom*

**A novel strategy combining peptide fingerprinting of single neurons by matrix-assisted laser desorption ionization mass spectrometry, molecular cloning, peptide chemistry, and electrospray ionization mass spectrometry was used to study the intricate processing pattern of a preprohormone expressed in identified neurons, the neuroendocrine light yellow cells (LYCs) of the gastropod mollusc, *Lymnaea stagnalis*. The cDNA encoding the precursor, named prepro-LYCP (LYCPs, light yellow cell peptides), predicts a straightforward processing into three peptides, LYCP I, II, and III, at conventional dibasic processing sites flanking the peptide domains on the precursor. However, matrix-assisted laser desorption ionization mass spectrometry of single LYCs revealed trimmed variant peptides derived from LYCP I and II. The variants were much more abundant than the intact peptides, indicating that LYCP I and II serve as intermediates in a peptide-processing sequence. Using the molecular masses of the peptides as markers to guide their isolation by well established purification methods, the structural identities of the peptides could be confirmed by amino acid sequencing. Furthermore, matrix-assisted laser desorption ionization mass spectrometry could detect colocalization of a novel peptide with the LYCPs.**

Bioactive peptides are commonly synthesized in the form of larger precursor proteins, from which the peptides are processed by the action of prohormone convertases (1). These endoproteases often cleave at dibasic amino acid sites, Lys-Arg or Arg-Arg, that flank the peptide domains (2, 3). Although these conventional processing sites and, therefore, putative peptides can be predicted from the precursor as encoded by the corresponding cDNA, independent biochemical evidence obtained by amino acid sequencing is needed, because unpredictable (tissue-specific) processing at unconventional cleavage sites as well as tissue-specific post-translational modifications may occur (4–8). However, the conventional methodology of peptide purification is cumbersome and often does not yield sufficient insight into the possibility of unpredictable processing steps. To circumvent this problem, we combined a novel technique, direct mass spectrometric analysis of peptides in single neurons, with well established molecular biological and peptide chemical techniques. We used cells from the snail *Lymnaea stagnalis*, a model neurobiological preparation that has been widely used for molecular, physiological, and behavioral studies (*e.g.* Refs. 9–12).

We examined the well characterized neuroendocrine light yellow cells (LYCs),[1] which occur in two clusters of about 10–25 neurons each in the visceral ganglion and the right parietal ganglion of *L. stagnalis*. The advantage of these neurons is that they can be easily recognized by visual inspection of the live ganglion, and single neurons can be dissected for analysis of their chemical content. The LYCs in the right parietal ganglion are electronically coupled neurons that generate synchronous bursts of spikes lasting about 20 min (13). *In vivo* recordings have demonstrated that the burst frequency increases both during egg laying and feeding,[2] indicating that these cells might be involved in the neuroendocrine regulation of these diverse behavioral processes. The cDNA that encodes the LYC peptide (LYCP) precursor (14) predicts that three peptides, named LYCP I, II, and III (Fig. 1), can be generated from the precursor by cleaving at conventional processing sites. LYCP II has previously been isolated and characterized (15). In addition, that study also identified a variant LYCP II with a trimmed single N-terminal amino acid residue. This variant peptide occurs in much higher amounts than LYCP II, suggesting that it is the mature end product. Because these results suggest a complex pattern of prepro-LYCP processing, involving cleavage sites that cannot be predicted from the cDNA data, we decided to investigate in detail the processing of the peptides by a novel strategy combining mass spectrometry, molecular cloning (14), and peptide chemistry. In short, we used matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) developed by Karas and Hillenkamp (16) to examine the peptide content of single LYCs. By comparing the measured masses of the peptides with their calculated masses as predicted by the corresponding cDNA, we were able to pinpoint the various steps in the unconventional processing of the LYCPs. We confirmed the MALDI-MS findings by both amino acid sequencing and electrospray ionization mass spectrometry

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§ To whom correspondence should be addressed: Faculty of Biology, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands. Fax: 31-20-444-7123.

[1] The abbreviations used are: LYCs, light yellow cells; ESI-MS, electrospray ionization mass spectrometry; LYCPs, light yellow cell peptides; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry.

[2] A. Ter Maat, personal communication.

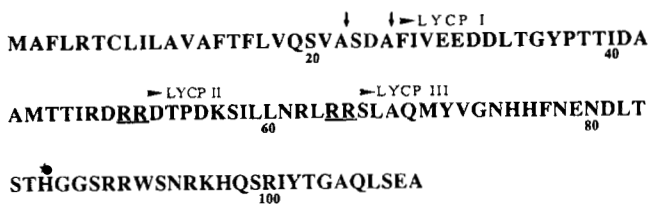


FIG. 1. **The organization and amino acid sequence of the LYCP precursor as deduced from cDNA cloning studies (14).** The putative dibasic cleavage sites that are predicted by the cDNA studies are *underlined*. The *arrows* indicate the two equally probable predicted signal sequence cleavage sites. The *asterisk* indicates the amino acid residue that was divergent in the present study (see "Results" for further details). *Numbers* indicate amino acid residue positions in prepro-LYCP.

(ESI-MS) of purified peptides. Furthermore, we found that a putative bioactive novel peptide, which is not generated from the LYCP precursor, colocalized with the LYCPs.

## MATERIALS AND METHODS

*MALDI-MS of Single LYCs*—Single LYCs taken randomly from the cluster of LYCs in the right parietal ganglion of mature laboratory-bred *L. stagnalis* were dissected under a microscope using tiny hooks. A glass pipette was used to remove and transfer the individual neurons to 1-μl drops of matrix solution (2,5-dihydroxybenzoic acid) on a stainless steel target. The solution was dried within minutes by a gentle stream of cool air, and the target was inserted into the mass spectrometer immediately afterwards. The acidic nature of the matrix (pH 2.0) generally inhibits the activities of the compartmentalized enzymes that might be released during cell lysing. MALDI-MS was performed on a Finnigan MAT Vision 2000 laser desorption time of flight mass spectrometer equipped with a pulse nitrogen laser. An external standard peptide, renin, was used for calibration. Usually, 30 individual spectra were accumulated to increase the signal:noise ratio.

*ESI-MS*—Fractions obtained by reversed phase high performance liquid chromatography were dried in a Speedvac and redissolved in 100 μl of 7.0 mM trifluoroacetic acid in 60% acetonitrile. ESI-MS of the peptide was performed as described (17) with minor modifications. About 8 μl of each fraction were injected via a 10-μl loop into a Fisons BioQ triple-quadrupole mass spectrometer equipped with an electrospray atmospheric pressure ionization source. The mobile phase was 50% acetonitrile; the flow rate was 5 μl/min.

*Peptide Purification*—The right internal pallial nerve (also known as right internal parietal nerve), which serves as one of the neurohemal areas of the LYCs (18), was used as starting material for purification of LYCPs. Nerves were dissected, collected on dry ice, and subsequently stored at −55 °C. Peptide extracts were made in 1 ml of 0.1 M acetic acid in 65% ethanol and centrifuged in an Eppendorf centrifuge (10 min). The extraction solvent of low pH and high alcohol content should stop enzyme activities. The supernatant was size fractionated using high performance gel permeation chromatography with Protein Pak I-125 and I-300 columns (Waters Associates) linked in series. The running solvent was 7.0 mM trifluoroacetic acid in 30% acetonitrile; the flow rate was 1 ml/min; and 1-min fractions were collected. We employed ESI-MS to screen the masses of peptides in the fractions in order to identify those fractions that contain putative LYCPs. Fractions having peptides that were not detected in MALDI-MS of single LYCs were not investigated. Fractions containing the putative LYCP I and III were separately pooled and further purified by reversed phase high performance liquid chromatography with Nucleosil C-18 columns (4.6 × 250 mm or 2.1 × 250 mm), using 7.0 mM trifluoroacetic acid as counterion and an increasing concentration of acetonitrile to elute the peptides.

*Endoprotease Digestion*—LYCP III was subjected to protease digestion using endoprotease Lys-C from Boehringer. The reaction was performed as recommended by the manufacturer, at room temperature for 16 h. The peptide with a mass of 4592 Da was digested by trypsin in a substrate:enzyme ratio of about 50:1 in phosphate-buffered saline at room temperature for 4 h. The reaction products were separated using a C18 column as described above, and the purified peptide fragments were subjected to amino acid sequencing.

*Amino Acid Sequencing*—Amino acid sequencing was done on an Applied Biosystems model 473 pulse liquid sequencer, using the sequencing program recommended by the company.

## RESULTS

*Peptide Fingerprinting of Single LYCs by MALDI-MS*—Single LYCs were isolated and ruptured, and individual cells were immediately subjected to MALDI-MS. The peptide patterns of the various LYCs tested were identical, and an example of the mass spectrum of one cell is given in Fig. 2. The mass spectrum reveals the presence of several peptide peaks. Assuming that the peptides are generated from a single LYCP precursor, these masses can be compared with those predicted by the cDNA cloning studies (14) (Fig. 1). These comparisons show that the masses of the two major peaks that were present in the 1–3-kDa range correspond to LYCP I and II variants with one amino acid trimmed at the N termini. Intact LYCP I and II were also detected; however, they were present at much lower levels than the trimmed peptides. Previously we have ruled out the possibility that the trimming of LYCP II was an artifact caused by the compartmentalized enzymes that were released after cell lysing by extracting the LYC clusters in acetic acid and acidified acetone separately (15). In the latter case all enzyme activities must be inhibited. Both procedures yielded similar results, *i.e.* more than 90% of the LYCP II existed as the N terminus trimmed form. This implies that low pH together with short sample handling time as had been the case in the present study, would be reliably used to prevent unwanted enzyme activity in this system. We can therefore conclude that the trimming of both LYCP I and II take place in the cell body. An intact putative LYCP III was present, but the mass was about 18 Da higher than the calculated mass based on the cDNA cloning studies. There is a peptide of 4592 Da that exists at a lower level than other LYCPs. This mass, however, does not correspond to the masses of predicted LYCPs, suggesting that the peptide is not the end product of the LYCP precursor. Generally, this study confirms the cDNA cloning studies that found that the LYCP precursor is the major gene product in the LYCs. The other major components in a cell such as phospholipids and small metabolites are either poorly extracted from the matrix and have low ionization efficiency[3] or are too small (<400 Da) to be effectively detected by the present method.

*Peptide Purification and Characterization*—In order to unequivocally confirm the identities of the intact LYCPs and their variants as revealed by MALDI-MS, we purified the peptides from the neurohemal area of the LYCs, the right internal pallial nerve, for biochemical characterization. Five hundred nerves were extracted and centrifuged, and the supernatant was size fractionated. Fractions were screened using ESI-MS to detect the presence of peptides with masses corresponding to those of the intact LYCPs and variants and the unknown peptide of 4592 Da detected in LYCs by MALDI-MS (Fig. 3). Peptides in the LYCs that were not detected by MALDI-MS (and apparently are produced by other types of neurons) were not investigated. Since LYCP II and its trimmed form had previously been isolated and characterized (15), we focused on LYCP I and III.

The LYCP III pool was resolved using a C18 column (Fig. 4), and mass measurements of the HPLC fractions by ESI-MS revealed the presence of a peptide of 5162 Da eluting at 32 min. Based on the data of single cell analysis by MALDI-MS this peptide was assigned as LYCP III. Indeed, partial amino acid sequencing of the peptide contained in the fraction revealed the N-terminal sequence of a peptide matching the predicted sequence of LYCP III. However, because of the relatively long sequence of the peptide, the recovery of phenylthiohydantoin-amino acid derivatives at the C-terminal region became so low that the sequence could not be unambiguously determined. In

[3] K. W. Li, unpublished results.

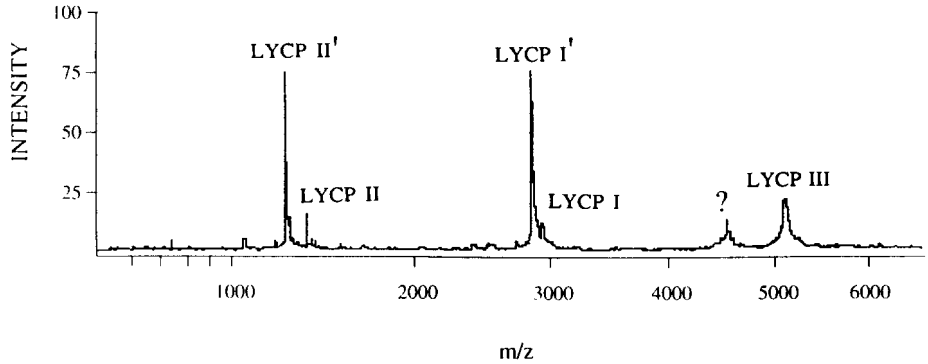


FIG. 2. **Mass spectrum of a single LYC using MALDI-MS.** Together five individual, randomly dissected LYCs from the cluster in the right parietal ganglion were studied. The mass spectra of these cells are identical, and in the figure only one example is presented. The calculated *versus* measured protonated masses are as follows: LYCP I, 2962.2 *versus* 2962.4 Da; N-terminal-trimmed LYCP I (*LYCP I'*), 2875.1 *versus* 2875.5 Da; LYCP II, 1385.6 *versus* 1386.6 Da; N-terminal-trimmed LYCP II (*LYCP II'*), 1270.5 *versus* 1271.6 Da; LYCP III, 5144.6 *versus* 5162.4 Da (see "Results" for details). The peptide peak with a *question mark* had a mass of 4592 Da and does not correspond to the mass of any predicted LYCP. *x* axis, *m/z* is mass to charge ratio; *y* axis, intensity in arbitrary units.

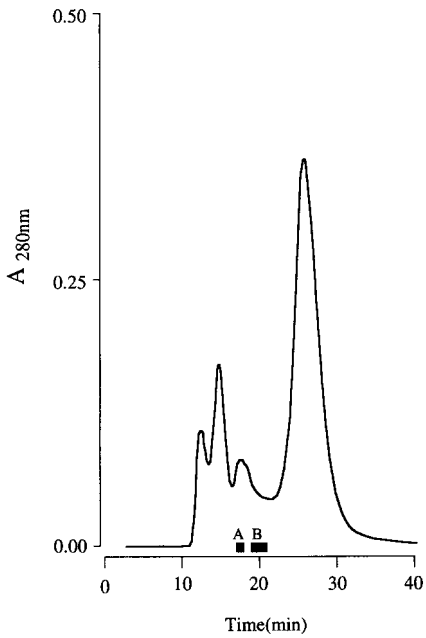


FIG. 3. **High performance gel permeation chromatography of right internal pallial nerve extract.** The masses of peptides in the fractions were screened by ESI-MS. Fractions corresponding to the *bar* labeled *A* contained several peptides including one with a mass corresponding to that of LYCP III. Fractions corresponding to the *bar* labeled *B* contained also various peptides including one with a mass corresponding to that of the LYCP I variant.

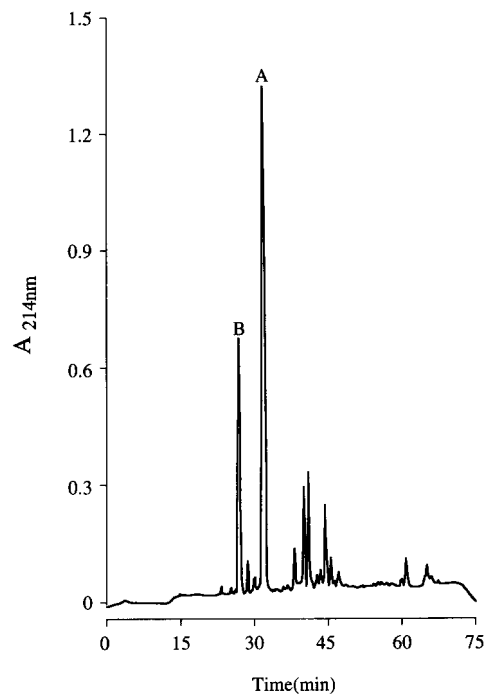


FIG. 4. **Reversed phase high performance liquid chromatography of LYCP III prepurified by gel permeation chromatography.** Fractions corresponding to the peak labeled *A* contained a peptide with a mass corresponding to that of LYCP III. Part of the fraction was subjected to amino acid sequencing, and the remaining fraction was used for endoproteinase Lys-C digestion. Fractions corresponding to the peak labeled *B* contained a molecule of 4592 Da. This molecule cannot be sequenced; however, trypsin digestion cleaved it into several fragments, indicating that it is a peptide, and so the resulting fragments were isolated and subjected to amino acid sequencing.

order to fully characterize the peptide, the rest of the fraction was treated with endoproteinase Lys-C, and the resulting peptide fragments were resolved using a C18 column. Amino acid sequencing of the peptides contained in the fractions corresponding to the two UV absorbance peaks revealed two peptide fragments representing residues 1–31 and 32–45 of LYCP III. Based on these data, the primary structure of the peptide is as follows: SLAQMYVGNHHFNENDLTSTRGGSRRWSNRKHQSRIYTGAQLSEA. The calculated mass based on the amino acid sequencing data is in agreement with the measured mass. However, residue 21 is Arg instead of the His predicted by the cDNA cloning studies. Since the difference represents a mass difference of about 19 Da, it explains the disparity between the measured mass and the predicted mass based on the cDNA studies (14).

Fig. 4 further shows a second major UV peak eluting at 27 min. ESI-MS revealed the presence of a peptide of 4592 Da in the right internal pallial nerve, which corresponds to the unknown peptide as detected in the soma of LYCs by using MALDI-MS single cell analysis. The fraction containing the molecule was subjected to amino acid sequencing, which gave no detectable phenylthiohydantoin-amino acid suggesting that it is not a peptide or that the peptide is N-terminally blocked. The molecule was further subjected to trypsin digestion, which resulted in the generation of several tryptic fragments, indicating that the molecule is a peptide. The fragments were resolved using a C18 column (data not shown), and amino acid sequencing of one of the fragments revealed the sequence YYKPAQTIQ, which does not resemble the amino acid sequence of LYCPs.

The LYCP I pool obtained by gel permeation chromatography

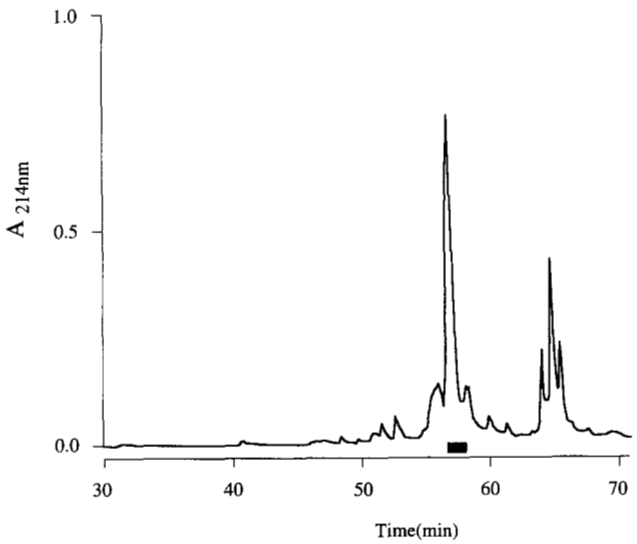


FIG. 5. **Purification of LYCP I.** Reversed phase high performance liquid chromatography of LYCP I prepurified by gel permeation chromatography is shown. Material in the fraction denoted by a *bar* contained LYCP I and its variant.

(Fig. 3) was further resolved using reversed phase high performance liquid chromatography (Fig. 5). ESI-MS of the fraction corresponding to the labeled peak in Fig. 5 revealed the presence of two peptides (Fig. 6), indicating that the two (closely related) peptides co-eluted in the reversed phase HPLC step. The most abundant peptide had a mass of 2875 Da, which is in agreement with the calculated mass of a truncated form of LYCP I lacking the N-terminal serine. The minor component had a mass of approximately 2962 Da, which corresponds to intact LYCP I with a calculated mass of 2962 Da. In order to confirm the proposed identities of the peptides, the fraction was subjected to amino acid sequencing, which revealed the presence of the truncated LYCP I with the amino acid sequence DAFIVEEDDLTGYPTTIDAAMTTIRD. The first residue contained also a low level of serine in addition to a very high level of aspartic acid. Since the only difference between the intact and the truncated LYCP I is the presence of an extra serine residue at the N terminus of the intact LYCP I, together with the mass spectrometric data, we conclude that a small amount of intact LYCP I with N-terminal SDAFIV was also present in the fraction.

## DISCUSSION

We used MALDI-MS peptide fingerprinting of single neuroendocrine cells in combination with the available cDNA cloning data (14) as a quick screening method to get an insight into the various steps involved in the processing of a prohormone. Because the use of MALDI-MS in peptide chemistry of single cells is novel, its value and reliability need to be rigorously tested by well established biochemical methods such as peptide purification, Edman degradation, and mass measurement of purified peptides. Therefore, we designed a strategy involving all of these methods and applied it to the neuroendocrine LYC system of *Lymnaea* that expresses the LYCP precursor. The LYC system has been intensively studied (13–15, 19), and the cDNA studies of Smit *et al.* (14) showed that prepro-LYCP contains three peptide domains that possibly could be straightforwardly processed to yield LYCP I, II, and III by cleaving off the signal sequence followed by processing events involving the conventional dibasic cleavage sites flanking the LYCP II domain. This suggestion was first examined by MALDI-MS of single LYCs, which enabled us to detect the masses of peptides that are present in the LYC cell bodies. The subsequent comparison of the measured peptide masses with their calculated masses predicted by the cDNA data then enabled us to deduce the complex processing pattern.

The initial event in the processing of all preprohormones is the removal of the hydrophobic signal sequence in the rough endoplasmic reticulum. As regards prepro-LYCP, the cleavage site used to remove the signal peptide appeared difficult to predict, since there are two equally probable sites in the preprohormone at residues 22 and 25 (14). Because we observed a peptide with a mass corresponding to a form of LYCP I with N-terminal SDAFIV (residues 23–49 of the preprohormone), we conclude that the first cleavage site (at residue 22) is used to remove the signal peptide from the LYCP precursor yielding pro-LYCP, which is the direct precursor to the LYCPs.

In general, a prohormone contains often multiple peptide domains, which are in most cases flanked by the dibasic cleaving sites, Lys-Arg or Arg-Arg, that are used by prohormone convertases to cleave out the peptides (1). In pro-LYCP, there are two such cleavage sites flanking the LYCP II domain. Because LYCP I, II, and III are detected in the mass spectrum, we conclude that these dibasic sites are indeed used. However, further processing of the peptides by the action of (unidentified) exopeptidases also appears to occur. This can be inferred from the occurrence of trimmed forms of both LYCP I (present study) and LYCP II (15). Since the intact and the trimmed peptides are structurally very similar, it is very likely that these peptides should have similar ionization efficiencies, in which case MALDI-MS should provide semiquantitative data about the abundance of the peptides (Ref. 20; see also Ref. 12). Since the MALDI-mass spectrum reveals that the variants are present at much higher levels than the intact peptides, which is also in agreement with the ESI-MS data, we conclude that they represent the end products; therefore, intact LYCP I and II must be the precursors of the variant peptides. Based on the mass spectrometric data, the putative processing pathway of prepro-LYCP can then be proposed as shown in Fig. 7.

The MALDI-MS data of single cell analysis was confirmed by using conventional peptide chemical methodology. We focused on LYCP I and its variant as well as on LYCP III. LYCP II and the derived variant were not investigated, since they have been previously identified by amino acid sequencing and shown to be present in different amounts (*i.e.* the variant peptide was present in much larger amounts than the intact peptide (15)). We first isolated the LYCP I variant from extracts and then chemically characterized the peptide. Interestingly, ESI-MS revealed that in the same fraction the LYCP I variant and the intact LYCP I are present, with the intact LYCP I present at a much lower level. The presence of these two peptides, in very different amounts, is in perfect agreement with the data of MALDI-MS of single LYCs.

The determination of the identity of LYCP III was problematic, because the MALDI-MS data indicated that the primary structure of LYCP III is not the same as the one predicted by the cDNA studies (14). This difference concerns residue 21 (residue 86 of the prepro-LYCP), which we identified as Arg instead of the predicted His. The difference fully explains the disparity between the present mass measurements and the mass based on the cDNA studies (which differ by about 18 Da; see "Results"). Again, this underscores the predictive value of MALDI-MS of single cell analysis.

In addition to the truncation of LYCPs reported in the present study, truncation of neuropeptides has been reported for the molluscan insulin-related peptides of *Lymnaea* (21–23) as well as for the $\alpha$ bag-cell peptide of *Aplysia* (24). In vertebrates, various insulins, relaxins, and insulin-like growth factors are

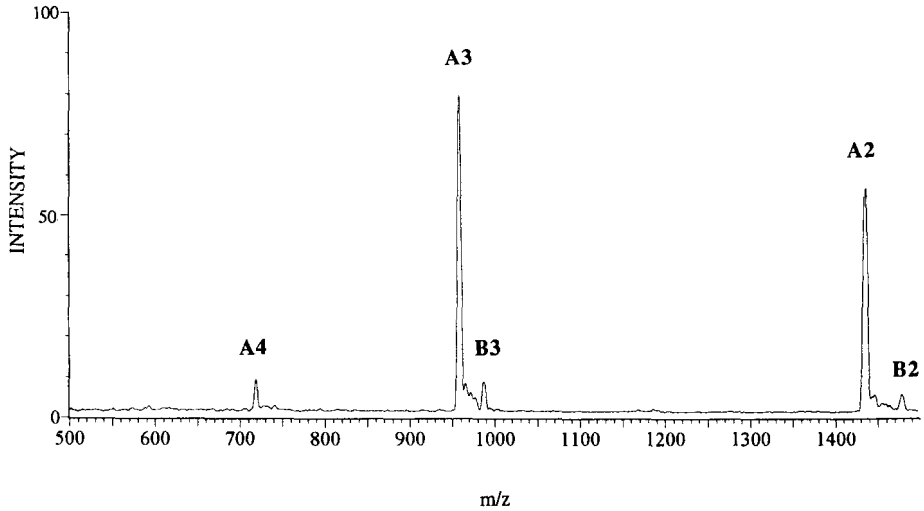


FIG. 6. **Mass spectrum of the purified LYCPs using ESI-MS.** *Peaks* of the *A*-series represent the multiple charged trimmed LYCP I. *Peaks* of the *B*-series represent the multiple charged intact LYCP I. *A2* and *B2*, double-protonated peptides; *A3* and *B3*, triple-protonated peptides; *A4*, quadruple-protonated peptide. The measured mass of trimmed LYCP I is 2875 Da, and that of intact LYCP I is 2962 Da. Intact LYCP I is present in the neurohemal area at a manifold lower level than trimmed LYCP I.

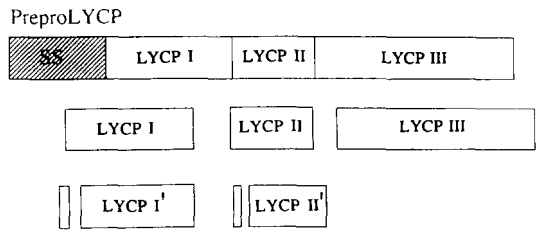


FIG. 7. **Proposed processing of prepro-LYCP.** The precursor is processed to yield the active neuropeptides by removal of the signal sequence (*SS*), followed by cleavage of dibasic sites as represented by *vertical bars*. LYCP I and II are further processed at their N termini to yield truncated mature peptides, indicated by LYCP I′ and LYCP II′.

post-translationally trimmed (25–27), and in some cases biological significance could indeed be attributed to this phenomenon (24).

Finally, MALDI-MS detected an additional putative bioactive peptide of 4592 Da in the LYCs. Because this mass does not correspond to the predicted mass of any of the LYCPs, it may represent the product of a different neuropeptide gene. This was confirmed by amino acid sequencing of the tryptic digest of the peptide isolated from the right internal pallial nerve, indicating that the primary structure of the peptide fragment does not bear any sequence similarity either to the peptide domains contained in pro-LYCP or to any other previously described molluscan neuropeptide. This result, therefore, shows that MALDI-MS analysis of single neurons and small samples of nervous tissue is a powerful tool for the detection of colocalized peptides.

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